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RESEARCH ARTICLE

Baolei WANG, Yi MA, Yonghong LI, Suhua WANG, Zhengming LI

The design, synthesis of amide KARI inhibitors and their biological activities

Abstract Ketol-acid reductoisomerase(KARI) is a promising target for the design of herbicides yet there are only few reports on the molecular design of KARI inhibitors. In this paper, based on the reported 0.165 nm high resolution crystal structure of the spinach KARI complex, 279 molecules with low binding energy toward KARI were obtained from an MDL/ACD 3D database search using the program DOCK 4.0. According to the structural information of 279 molecules provided, some amide compounds have been designed and synthesized. The bioassay results show that most of these amides had inhibitory activity to rice KARI at a test concentration of 200 μg/mL. Among which eight amides, compounds **1** and **6** show 57.4% and 48.1% inhibitory activity to KARI. The herbicidal activities of these amides were further investigated on di-cotyledonous rape (*Brassica campestris*) and mono-cotyledonous barnyardgrass (*Echinochloa crusgalli*). Compounds **1** and **6** were more favorable than others and showed 52.0% and 72.6% inhibitory activity on rape root at 100 μg/mL concentration, respectively. These amides could be further optimized for finding more potent candidates.

Keywords KARI, inhibitor, molecular design, biological activity

Ketol-acid reductoisomerase (KARI, EC 1.1.1.86), also known as acetohydroxy acid isomeroreductase (AHIR), is one of the key enzymes for the synthesis of branched-chain amino acids (isoleucine, leucine and valine). There are reviews proposing KARI as a herbicidal target [1]. Although the molecular design of potential KARI inhibitors is in accord with green herbicidal research, there are few reports of new KARI inhibitors except Hoe 704, IpOHA, 1,2,3-thiadiazoles and CPD derivatives, which are potent inhibitors of the enzyme only *in vitro,* but their activities *in vivo* as herbicides are weak [2–4].

In our previous work [5], 279 molecules with low combining energy toward KARI were obtained by an MDL/ACD-3D database search using the DOCK 4.0 program based on the crystal structure of the KARI complex, which provides us plentiful structural information for our research. Many amides were found among the 279 molecules. In looking for novel potent KARI inhibitors, several types of amides were synthesized and their herbicidal activities both *in vitro* and *in vivo* were investigated. The novel structures are shown in Fig. 1.

1 Experiments

1.1 Apparatus and materials

The DOCK (version 4.0) program was employed. The computational work was based on the 0.165 nm crystal structure of the spinach KARI complex (PDB code 1YVE) and finished on the SGI Indigo R10000 of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences and SCI Indigo 2 workstations of State Key Laboratory of Elemento-Organic Chemistry, Nankai University. The MDL/ACD 3D database of 250000 organic compounds was searched for potential KARI inhibitors.

Melting points were determined using a Taike X-4 apparatus and were uncorrected. The infrared spectra were recorded on a BRUCK EQUINOX55 FTIR spectrophotometer as KBr tablets. ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AV-300 instrument (300 MHz) using TMS as an internal standard. Mass spectra were recorded on a Hewlett Packard G1800A and an HP-1100 LC/MS instruments. Elemental analyses were performed on a Yanaco MT-3CHN elemental analyzer.

NADPH was obtained from Sigma and hydroxypyruvate was obtained from Fluka. 4-Methoxybenzoyl chloride and *N*-(2-bromoethyl)phthalimide were synthesized by routine methods. Other reagents were of AR pure grade and were used directly.

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Baolei WANG, Yi MA, Yonghong LI, Suhua WANG,

Zhengming LI (✉)

Elemento-Organic Chemistry Institute, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, China
E-mail: nkzml@vip.163.com

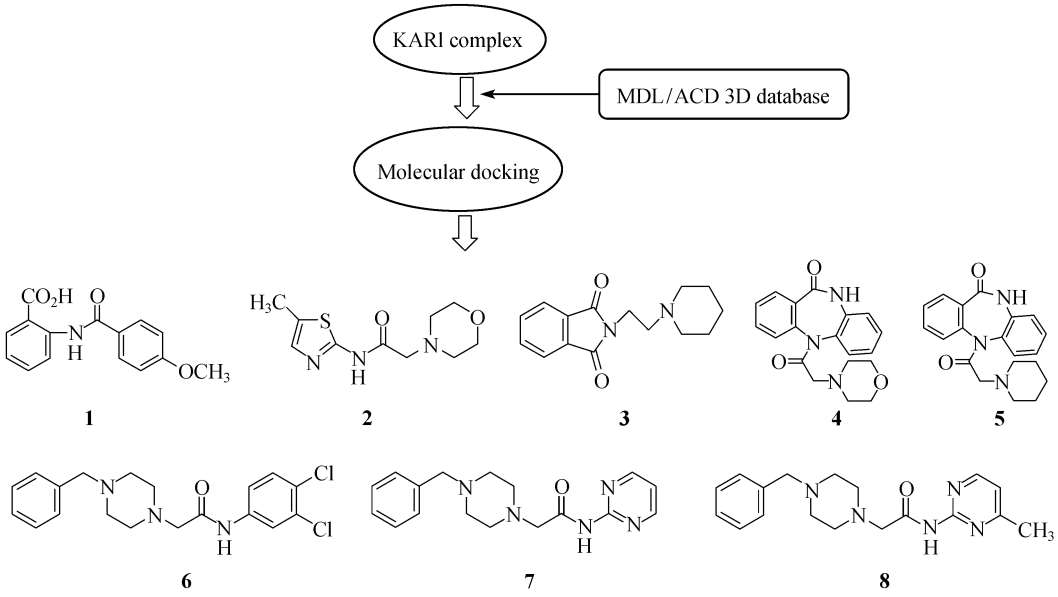

Fig. 1 Structures of designed compounds

1.2 Database searching

Based on the 0.165 nm crystal structure of spinach KARI-IpOHA complex (PDB code 1YVE) [6], some preparation works, *i.e.*, ligand preparation, site characterization and scoring grid calculation were consequently completed. Then, molecular docking was run to search the MDL/ACD 3D database of 250000 organic compounds using the DOCK 4.0 program. 279 molecules, as potential KARI inhibitors, were thus obtained after the database search [5]. In this paper, several amides among the 279 molecules were synthesized and investigated for the KARI inhibitory and herbicidal activities.

1.3 Synthesis

1.3.1 2-(4-Methoxybenzamido)benzoic acid (**1**)

To a well-stirred mixture of 2-aminobenzoic acid (1.37 g, 0.01 mol), triethylamine (1.11 g, 0.011 mol) and 20 mL dry toluene, 4-methoxybenzoyl chloride (1.71 g, 0.011 mol) was added dropwise. After being stirred at room temperature for 2 h, the mixture was acidified with diluted hydrochloric acid. The solid was filtered out and recrystallized with ethanol/water to give 1.9 g of compound **1** as a white crystal, mp 233–235°C, yield 70%; ^{1}H-NMR (DMSO-d_6), δ: 12.15 (s, 1H, –CO_2H), 8.75–7.12 (m, 8H, Ph–H), 3.86 (s, 3H, OCH_3); Elemental analysis (%, calcd. for $C_{15}H_{13}NO_4$): C 66.19 (66.41), H 4.77 (4.83), N 5.34 (5.16).

1.3.2 2-[2-(4-Morpholino)]acetamido-4-methylthiazole (**2**)

To an ice-bath, cooled and stirred mixture of 2-amino-5-methylthiazole (1.14 g, 0.01 mol), anhydrous K_2CO_3 (1.38 g, 0.01 mol) and 40 mL dry toluene, a solution of chloroacetyl chloride (1.13 g, 0.01 mol) in 5 mL dry toluene was added dropwise and further stirred at room temperature overnight. The mixture was filtered and the solid was washed with water, then recrystallized with chloroform/petroleum ether to give 1.25 g of 2-chloroacetylamino-5-methylthiazole (intermediate) as a white crystal, mp 167–170°C, yield 66%; ^{1}H-NMR ($CDCl_3$), δ: 7.12 (br, 1H, NH), 4.23 (s, 1H, Thiazole–H), 2.41 (s, 2H, CH_2), 2.15 (s, 3H, CH_3).

The intermediate (0.38 g, 2 mmol) was heated to solve in 25 mL acetone. To this solution, morpholine (0.17 g, 2 mmol) and anhydrous K_2CO_3 (0.28 g, 2 mmol) were added. The mixture was stirred and refluxed for 6 h. After being cooled down to room temperature, the mixture was condensed. The residue was poured into water and stirred for 5 min. The solid was then filtered and washed with water to give 0.25 g of compound **2** as a white crystal, mp 141–144°C, yield 52%. The product can be further purified by recrystallization with ethanol/water, mp 143–144°C; ^{1}H-NMR ($CDCl_3$), δ: 10.26 (br, 1H, NH), 7.11 (s, 1H, Thiazole–H), 3.79 (t, J = 3.6 Hz, 4H, –NCH_2CH_2O–), 3.26, 3.25 (d, 2H, –$NHCOCH_2$–), 2.64 (t, J = 3.6 Hz, 4H, –NCH_2CH_2O–), 2.43 (s, 3H, CH_3); IR (KBr), v: 3292 (N–H), 1721 (C = O), 1587 (C = N); ESI-MS, m/z: 242 (M + 1); Elemental analysis (%, calcd. for $C_{10}H_{15}N_3O_2S$): C 49.51 (49.77), H 6.23 (6.27), N 17.13 (17.41).

1.3.3 N-(2-(piperidin-1-yl)ethyl)phthalimide (**3**)

To a well-stirred mixture of piperidine (0.13 g, 1.5 mmol), anhydrous K_2CO_3 (0.21 g, 1.5 mmol) and 10 mL toluene, a solution of *N*-(2-bromoethyl)phthalimide (0.38 g,

1.5 mmol) in 5 mL toluene was added dropwise. After being stirred and refluxed for 3 h, the mixture was cooled down to room temperature and filtered, and the solid was washed with toluene. The filtrate was combined and the solvent was removed under reduced pressure. The residual yellow oil was purified by silica gel chromatography with petroleum ether and ethyl acetate (volume ratio 8: 1–5: 1) as solvents and followed by recrystallization with petroleum ether to give compound **3** as a yellow crystal, yield 68%, mp 83–85°C (Ref. [7] mp 89–90°C).

1.3.4 5-[(4-Morpholino/piperidin-1-yl)acetyl]-10, 11-dihydro-5H-dibenzo[b,e][1,4]diazepin-11-one (**4**, **5**)

1,2-Diaminobenzene (5.41 g, 0.05 mol), 2-chlorobenzoic acid (7.83 g, 0.05 mol) and 175 mL chlorobenzene were placed in a 500 mL four necked flask equipped with mechanical stirrer and condenser. After the mixture was heated to be solved, copper powder (9.53 g, 0.15 mol) and 5Å molecular sieve (3.50 g) were added to it. The mixture was refluxed at 130°C for 8 h with stirring and then filtered immediately. The solvent was removed and the residual solid was recrystallized with ethanol to give 5.02 g of 11-oxo-10,11-dihydro-5*H*-dibenzo[b,e][1,4]diazepine as a yellow crystal, yield 48%, mp 254–256°C (Ref. [8] mp 256–257°C).

Diazepine prepared above reacted with chloroacetyl chloride in THF using *N*,*N*-dimethylaniline as acid acceptor to give 5-(2-chloroacetyl)-10,11-dihydro-dibenzo[b,e][1,4]diazepin-11-one as a white crystal, yield 53.8%, mp 240–241°C (Ref. [9] mp 241–242°C).

The diazepinone intermediate (0.6 g, 2.1 mmol) was heated to solve in 50 mL acetonitrile. To this solution, morpholine or piperidine (2.4 mmol) and anhydrous K_2CO_3 (0.2 g, 1.45 mmol) were added. The mixture was stirred and refluxed for 5 h. After being cooled down to room temperature, the mixture was distilled to remove the solvent under reduced pressure. 20 mL water was then added to the residue with stirring. The mixture was extracted with ethyl acetate (15 mL×4) and dried over magnesium sulfate. The ester was removed and the residue was recrystallized with ethyl acetate to give the target compound as a white crystal.

4: yield 48%, mp 190–192°C; ^{1}H-NMR ($CDCl_3$), δ: 9.73, 9.09 (d, 1H, NH), 8.01–7.24 (m, 8H, Ph–H), 3.63 (m, 4H, $-OCH_2-$), 3.20 (t, 2H, $-CH_2CO-$), 2.45–2.31 (m, 4H, $-NCH_2CH_2-$); IR (KBr), *v*: 3283 (N–H), 1690, 1671 (C = O); ESI-MS, *m/z*: 338 (M + 1); Elemental analysis (%, calcd. for $C_{19}H_{19}N_3O_3$): C 67.35 (67.64), H 5.84 (5.68), N 12.17 (12.46).

5: yield 60%, mp 198–200°C; ^{1}H-NMR ($CDCl_3$), δ: 9.30, 8.69 (d, 1H, NH), 8.00–7.20 (m, 8H, Ph–H), 3.17(t, 2H, $-CH_2CO-$), 2.35–2.17(m, 4H, $-NCH_2CH_2-$), 1.47–1.32 (m, 6H, $-NCH_2CH_2CH_2-$); IR (KBr), *v*: 3312 (N–H), 1683, 1669 (C = O); ESI-MS, *m/z*: 336 (M + 1); Elemental analysis (%, calcd. for $C_{20}H_{21}N_3O_2$): C 71.55 (71.62), H 6.58 (6.31), N 12.33 (12.53).

1.3.5 2-(4-Benzylpiperazin-1-yl)-N-arylacetamide (**6**–**8**)

N-benzylpiperazine was synthesized according to the literature [10]; intermediates *N*-(3,4-dichlorophenyl)chloroacetamide, *N*-(pyrimidin-2-yl)chloroacetamide and *N*-(4-methylpyrimidin-2-yl)chloroacetamide were synthesized according to the literature [11].

The intermediate prepared above (3 mmol) was heated to solve in 20 mL toluene. To this solution, *N*-benzylpiperazine (3 mmol) and triethylamine (0.32 g, 3.2 mmol) were added at room temperature. The mixture was stirred and refluxed for 5 h. After being cooled down to room temperature, the mixture was filtered and the filtrate was distilled to remove the solvent under reduced pressure. The residual solid was purified by recystallization or silica gel chromatography to give the target compound.

6: The compound was obtained in 57% yield as a white crystal by recrystallization with ethanol/water, mp 111–112°C; ^{1}H-NMR ($CDCl_3$), δ: 9.20 (s, 1H, NH), 7.77–7.30 (m, 8H, Ph–H), 3.59 (s, 2H, $-CH_2CO-$), 3.15 (s, 2H, Ph–CH_2-), 2.68–2.58 (m, 8H, Piperazine–H); Elemental analysis (%, calcd. for $C_{19}H_{21}Cl_2N_3O$): C 60.18 (60.32), H 5.41 (5.60), N 11.09 (11.11).

7: the compound was obtained in 45% yield as a light yellow crystal by silica gel chromatography with ethyl acetate and petroleum ether (volume ratio 9: 1) as solvents, mp 119–121°C; ^{1}H-NMR ($CDCl_3$), δ: 9.89 (s, 1H, NH), 8.67, 8.65 (d, J = 4.8 Hz, 2H, Pyrimidine–$H_{4,6}$), 7.33–7.32 (m, 5H, Ph–H), 7.05 (t, J = 4.8 Hz, 1H, Pyrimidine–H_5), 3.56 (s, 2H, $-CH_2CO-$), 3.22 (s, 2H, Ph–CH_2-), 2.68–2.58 (m, 8H, Piperazine–H); ^{13}C NMR ($CDCl_3$), δ: 168.43 (C = O), 162.31, 158.47, 157.11, 116.82 (PyrimidineC), 137.83, 129.13, 128.27, 127.17 (PhC), 62.82 ($PhCH_2$), 62.31 (CH_2CO), 53.46, 52.99 (PiperazineC); IR (KBr), *v*: 3288 (N–H), 1716 (C = O), 1574 (C = N), 1497, 1447 (C = C); GCD-MS, *m/z*: 311 (M^+), 189, 122, 91; Elemental analysis (%, calcd. for $C_{17}H_{21}N_5O$): C 65.23 (65.57), H 6.74 (6.80), N 22.19 (22.49).

8: the compound was obtained in 43% yield as a light yellow crystal by silica gel chromatography with ethyl acetate as solvent, mp 140–142°C; ^{1}H-NMR ($CDCl_3$), δ: 9.73 (s, 1H, NH), 8.52, 8.50 (d, J = 5.1 Hz, 1H, Pyrimidine–H_6), 7.34–7.33 (m, 5H, Ph–H), 6.91, 6.89 (d, J = 5.1 Hz, 1H, Pyrimidine–H_5), 3.58 (s, 2H, $-CH_2CO-$), 3.21 (s, 2H, Ph–CH_2-), 2.68–2.59 (m, 8H, Piperazine–H), 2.51 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$), δ: 168.42 (C = O), 162.36, 157.93, 156.94, 116.47 (PyrimidineC), 137.75, 129.17, 128.29, 127.21 (PhC), 62.84 ($PhCH_2$), 62.41 (CH_2CO), 53.46, 52.96 (PiperazineC), 24.19 (CH_3); IR (KBr), *v*: 3266 (N–H), 1727 (C = O), 1584 (C = N), 1502, 1437 (C = C); GCD-MS, *m/z*: 325 (M^+), 189,

Table 1 Biological activities data of compounds (% inhibition)

Compd.	Activity on rice KARI in 200 μg/mL conc. (% inhibition)	Herbicidal activity (% inhibition)			
		Rape root test (*brassica campestris*)		Barnyardgrass cup test (*echinochloa crusgalli*)	
		10 μg/mL	100 μg/mL	10 μg/mL	100 μg/mL
1	57.4	10.3	52.0	8.4	25.9
2	32.3	2.9	10.2	3.7	14.1
3	36.4	0	2.7	7.8	34.3
4	0	0	0	0	9.9
5	5.7	0	0	0	0
6	48.1	8.2	72.6	5.1	28.3
7	10.6	0	0	0	9.5
8	13.9	0	0	0	13.2

91; Elemental analysis (%, calcd. for $C_{18}H_{23}N_5O$): C 66.31 (66.44), H 7.05 (7.12), N 21.38 (21.52).

2 Results and discussion

2.1 Synthesis and ^{1}H-NMR spectra

Dibenzodiazepinone was synthesized by the reaction of 1,2-diaminobenzene and 2-chlorobenzoic acid using copper powder as catalyst *via* Ullmann-Golgber condensation. It was reported that the use of a molecular sieve will raise the yield of the product. However, the concrete quantity was unknown in the reference [8]. In our experiments, we found that the condensation product can be obtained in a 48% yield in the presence of 5 Å molecular sieve and a 3: 1 mole ratio of copper and 2-chlorobenzoic acid. Other intermediates and target compounds were synthesized according to the routine methods of corresponding literatures. (Some compounds existed in MDL/ACD database, but the characterization of their structures was not mentioned in the literature, so their structures were characterized further in this paper.)

In the ^{1}H-NMR spectra of compound **4**, the –NHCO–proton signals were observed at $\delta = 9.73$ and $\delta = 9.09$ as a doublet, $–CH_2CO–$ protons were split into a triplet, which are similar to the case of compound **5**. Moreover, the –NHCO– proton in seven-member ring of the intermediate was also appeared at $\delta = 9.13$ and $\delta = 8.77$ as doublet. All these can be owing to the existence of spin isomers in amide compounds.

2.2 Biological activities

The cloning of rice KARI has been described previously [12], and enzyme expression and purification followed that protocol. KARI activity was measured with a continuous assay method [13], following the consumption of NADPH at 340 nm and 30°C. The assay solution contained 0.2 mmol/L NADPH, 1 mmol/L $MgCl_2$, 0.1 mmol/L substrate (2-acetolactate), and inhibitors(**1**–**8**), in 0.1 mol/L phosphate buffer(pH 8.0). Inhibitors were preincubated with the enzyme in a phosphate buffer at 30°C for 10 min. The reaction was then started by adding the substrate combined with NADPH and $MgCl_2$. The percentage of the inhibition was calculated. The herbicidal activity of the compounds was determined on di-cotyledonous rape (*Brassica campestris*) and mono-cotyledonous barnyardgrass (*Echinochloa crusgalli*) by rape root and barnyardgrass cup tests, respectively, according to the literature [14]. *In vitro* biological assay data on rice KARI and herbicidal activity data of the compounds tested are listed in Table 1.

From Table 1, the bioassay results show that most of these amides had inhibitory activity to rice KARI at a test concentration of 200 μg/mL. Among which eight amides, compounds **1** and **6** showed 57.4% and 48.1% inhibitory activity to KARI. From the herbicidal activity data listed in same Table 1, compounds **1** and **6** were more favorable than others and show 52.0% and 72.6% inhibitory activity on rape root at 100 μg/mL concentration, which correlated with their *in vitro* activity to KARI. These amides could be further optimized for more potent KARI inhibitors.

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